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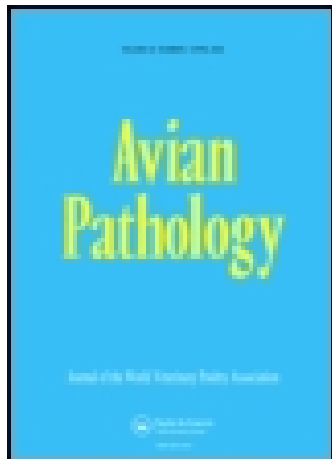
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Identification and characterization of avian hepatitis E virus in 2013
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Running title: Hepatitis E virus associated with disease

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Abstract

Two commercial Midwestern egg-type chicken flocks experienced significant increases in mortality rates in April 2013 with clinical signs appearing in 17-week-old pullets in Farm A and in 46-week-old hens in Farm B. Average weekly mortality was 0.44% over a 4-week period in Farm A and 0.17% over an 8-week period in Farm B. On Farm A, flocks in the affected house had a 45% decrease in daily egg production from weeks 19 to 27 when compared to standard egg production curves ($p < 0.01$) while no decrease in egg production was noticed in Farm B. Postmortem examination revealed changes consistent with hepatitis-splenomegaly syndrome, including hepatomegaly with serosanguineous fluid in the coelomic cavity and hepatic subcapsular hemorrhages. Microscopic lesions were characterized by multifocal necrotizing hepatitis and intrahepatic hemorrhage. No significant bacteria were recovered from liver samples, but 72.0-100% of the liver samples from affected chickens in Farm A (8/11) and Farm B (7/7) contained detectable amounts of avian hepatitis E virus (aHEV) RNA as determined by polymerase chain reaction. Sequencing and phylogenetic analysis of a 361-base-pair fragment of the helicase gene demonstrated 98.6-100% nucleotide identity between the aHEV genomes from Farm A and Farm B, whereas identities ranged from 74.6-90.5% when compared to other representative sequences. Sequences from this study clustered within aHEV genotype 2 previously recognized in the USA. In contrast to other reported aHEV outbreaks which occurred in 30 to 80-week-old-chickens, in the present investigation clinical aHEV was identified in 17-week-old chickens in one of the farms.

Introduction

Hepatitis E virus (HEV) is a member of the genus *Hepevirus* within the family *Hepeviridae*, which are non-enveloped, single-stranded RNA viruses with icosahedral symmetry consisting of at least four recognized genotypes of mammalian HEV (mHEV) and a separate floating species consisting of four genotypes of avian HEV (aHEV) (Bilic *et al.*, 2009; Marek *et al.*, 2010; Banyai *et al.*, 2012; Meng *et al.*, 2012; Hsu and Tsai *et al.*, 2014). Phylogenetic analysis of the full or nearly complete genome of aHEV strains indicated the presence of genotype 1 in Australia and Korea, genotype 2 in the USA, genotype 3 in Europe and China and, more recently, a novel genotype 4 has been described in Hungary and Taiwan (Bilic *et al.*, 2009; Zhao *et al.*, 2010; Kwon *et al.*, 2012; Banyai *et al.*, 2012; Hsu and Tsai, 2014). Additional putative genotypes comprising isolates from North America and Europe have been identified through analysis of a 130 nt fragment of the helicase gene, suggesting that diversity within aHEV could be higher (Marek *et al.*, 2010).

Avian HEV was associated with big liver and spleen disease (BLSD) in Australia (Payne *et al.*, 1999) and hepatitis-splenomegaly syndrome (HSS) in North America (Haqshenas *et al.*, 2001). Subsequently, aHEV infection has been associated with disease outbreaks in chicken flocks worldwide (Huang *et al.*, 2002; Agunos *et al.*, 2006; Morrow *et al.*, 2008; Bilic *et al.*, 2009; Zhao *et al.*, 2010; Marek *et al.*, 2010; Sprygin *et al.*, 2012). Chickens affected by HSS typically present enlarged livers, enlarged spleens, and serosanguineous fluid in their coelomic cavities accompanied by a drop in egg production and high mortality rates (Meng *et al.*, 2013). Characteristic histopathological changes may include massive coagulative necrosis, vasculitis, hemorrhage, amyloid deposition and non-specific hepatitis with a wide distribution of the aHEV through the parenchyma of liver tissue (Agunos *et al.*, 2006; Morrow *et al.*, 2008).

Based on serological evidence, aHEV is widespread in chicken flocks with seropositive rates of approximately 71% in the USA, 90% in Spain, and 57% in Korea (Huang *et al.*, 2002; Peralta *et al.*, 2009a; Kwon *et al.*, 2012). However, the role of aHEV in HSS is unclear, as the virus has also been detected in flocks with no history of HSS (Sun *et al.*, 2004; Peralta *et al.*, 2009b; Kwon *et al.*, 2012). Differences in virus strain, virus dose, diet and age have been implicated as potential co-factors for the manifestation of the full spectrum of clinical HSS (Agunos *et al.*, 2006; Meng *et al.*, 2013). Nevertheless, aHEV strains recovered from healthy chickens in normal flocks and previously considered to be avirulent were only slightly attenuated in an experimental infection model (Billam *et al.*, 2009). There was no clear clustering of aHEV sequences based on whether viruses were sampled from chickens displaying clinical signs or not (Marek *et al.*, 2010).

The present report describes field outbreaks of HSS in two laying hen flocks during spring 2013 in the USA and documents the clinical, pathological and microbiological findings in addition to aHEV genetic characteristics.

Materials and Methods

Farm and clinical observations. In April 2013, disease outbreaks with elevated mortality were observed on two laying hen farms located in the Midwestern USA. Affected flocks in both farms consisted of white laying hens vaccinated against Marek's disease, infectious bronchitis, Newcastle disease, avian influenza, fowl pox, *Mycoplasma gallisepticum*, and *Salmonella* Enteritidis. Vaccination against *Salmonella* Enteritidis on both farms consisted of administration

of a live attenuated *Salmonella* Typhimurium vaccine via the water followed by intramuscular injection of killed *Salmonella* Enteritidis in an oil emulsion at 12 weeks of age. This vaccination protocol is typical of programs utilized by Midwest egg producers in the USA.

Farm A was a 1,000,000 commercial laying hen facility, with an average house capacity of 111,000 white birds and a total of 9 houses. Each house on Farm A was managed on the basis of all-in, all-out principles and received replacement pullet flocks from a single source. Unusually high mortality associated with a decline in food and water consumption was observed in 1 of 9 houses after placement of a new lot of 16-week-old pullets. Weekly mortality peaked (0.78%) at 19 weeks of age and thereafter declined to normal levels (0.08%) during the ensuing 3 weeks (Fig. 1A). Weekly mortality in the remaining 8 unaffected houses continued to remain below 0.15% during this period. Egg production, which normally begins at 18 weeks, was delayed for 2 weeks in the affected pullet flock and did not begin until the flock reached 20 weeks of age. Egg production was reduced by 45% during the 8 week period from 19 to 27 weeks of age, after which time the flock's performance returned to near the breed standard (Fig. 1A). No other clinical signs of disease were noted.

Farm B had 1,200,000 commercial white laying hens of a different breed than those in Farm A and was located in a different state than Farm A. Farm B had an average house capacity of 150,000 birds and a total of 8 houses. Houses on this farm were also operated on all-in, all-out principles. On Farm B, increased mortality was observed in a single house containing 46-week-old hens. Maximum weekly mortality increased from an average of 0.09 to 0.17% in the affected flock, peaked (0.19%) at 48 weeks of age, and thereafter declined to 0.13% during the following 13 weeks (Fig. 1B). Egg production in Farm B remained within normal parameters and no other

clinical signs were observed. Weekly mortality in the remaining 7 unaffected houses continued to remain below 0.15% during this period and egg production was not affected (Fig. 1B).

Sample collection, histopathology, and bacteriology. Six 20-week-old dead chickens from the affected house on Farm A and five 46-week-old dead chickens from Farm B were submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for diagnostic workup. In addition, 6 weeks after the initial outbreak on Farm A livers were collected from 12 23-week-old dead chickens were from the affected house. Part of each liver was fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, embedded in paraffin, sectioned at 4 microns thickness, stained with hematoxylin and eosin and Congo red stain, and examined via light microscopy. Samples of the liver and spleen were cultured on agar plates using routine procedures under aerobic and anaerobic conditions at the ISU-VDL. In addition, samples were cultured in tetrathionate broth medium in an attempt to isolate *Salmonella* sp. The remaining portions of fresh livers were stored at -80°C until processed for virological analysis.

Virology. Liver samples of approximately 1 gram were minced and diluted 1:10 in phosphate-buffered saline (PBS), homogenized by using a Stomacher® 80 (Seward Laboratory Systems Inc, Bohemia, NY, USA), and centrifuged at $1,500 \times g$ for 10 min to obtain supernatant. All samples were stored at -80°C until usage. RNA extractions on liver homogenates were performed using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). Extracts were subsequently used for detection of the helicase gene of aHEV using primers previously described (Sun *et al.*, 2004) in a nested reverse transcriptase polymerase chain reaction (RT-PCR) reaction. Briefly, external primer set 5'-TGTTATYACACCCACCAARACGYTG-3', and 5'-

CCTCRTGGACCGTWATCGACCC-3'; and internal primer set 5'-GCCACGGCTRTTACACCYCA YGT-3', and 5'-GACCCRGGRITTCGACTGCTT-3' were used. Reverse transcriptase reaction and first round PCR were performed with OneStep RT-PCR Kit (Qiagen) according to manufacturer's instructions under the following conditions: 50°C for 30 min, 95°C for 15 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by a final elongation step of 72°C for 10 min. The second round PCR was performed with the ReadyMix® Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA) under the following conditions: 95°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final elongation step at 72°C for 7 min. PCR products with the expected size (386 bp) were examined on a 1% agarose gel, excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Two pools of three liver samples each from 20-week-old chickens from Farm A and five liver samples from Farm B were submitted to the University of Georgia Veterinary Diagnostic Laboratory (UGA-VDL) for adenovirus detection.

Sequencing and phylogenetic analysis. Sequencing of at least two aHEV RNA positive samples from each farm was performed directly on both strands at the ISU DNA Facility, Ames, Iowa, USA. Sequences were aligned with published data using BLAST at the national Centre for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>). Sequences were compiled using Lasergene software and the Clustal V alignment algorithm (DNASTar, Madison, WI, USA). Identical nucleotide sequences were represented as one sequence and used in phylogenetic analysis. For sequence analysis, the 361 bp sequences of the helicase gene were compared to each other and to sequences of aHEV isolates from Australia (GenBank Accession No. AM943647) and South Korea HH-F9 (GenBank Accession No. JN597006) representing

genotype 1, the USA prototype (GenBank Accession No. AY535004), USA avirulent (GenBank Accession No. EF206691), and Spain aHEVSP-46 (GenBank Accession No. EU919187) representing genotype 2, aHEV isolates from Europe (GenBank Accession No. AM943646) and China (GenBank Accession No. GU954430) representing genotype 3, and isolates from Hungary (GenBank Accession No. JN997392) and Taiwan (GenBank Accession No. KF511797) representing genotype 4. The nucleotide distance of the sequences was evaluated by neighbor-joining (NJ) using Lasergene MegAlign. Confidence in the NJ tree was estimated by bootstrap replicates. Sequences reported in this paper have been deposited in the GenBank database under the accession numbers KF573195 to KF573200.

Results

Post-mortem examination. Macroscopic examination of 20-week-old dead chickens from Farm A and 46-week-old dead chickens from Farm B revealed unusually dark livers with hepatomegaly and splenomegaly in all submitted chickens. Feather cover on these chickens was normal and no other macroscopic abnormalities were observed. Examination of 23-weeks-old dead chickens from the affected house on Farm A, collected after mortality levels had declined to normal levels, revealed that 5/12 had severely enlarged livers (Fig. 2A), 2/12 had livers that were moderately larger than normal, and in the remaining 5/12 chickens, livers were normal in appearance. Enlarged livers were friable and stippled with red, yellow, or tan foci with frequent subcapsular hemorrhages (Fig. 2B). In 4/5 chickens with severe liver lesions, approximately 15 ml clear straw-colored or blood-tinged fluid was present in the coelomic cavity. In these chickens

skeletal muscles were pale (Fig. 2C). To investigate possible causes for the presence of the coelomic cavity fluid, keel bones and rib bones were examined and fractures were not observed.

Histopathological examination. Grossly affected livers were microscopically characterized by multifocal moderate-to-severe necrotizing hepatitis with large, multifocal-to-coalescing areas of hepatocellular necrosis and hemorrhage (Fig. 3). Amorphous eosinophilic material was present in the space of Disse between the Kupffer cells and the hepatocytes. Liver sections stained with Congo red and viewed with polarized light did not show apple-green birefringence associated with amyloid. In acutely affected livers, low numbers of inflammatory cells (heterophils) were present. In subacutely infected livers, large numbers of dead and dying heterophils and pools of condensed fibrin were present in affected areas. Hepatocytes did not have increased cytoplasmic deposition of fat.

Bacteriology. Low numbers of *Escherichia coli* were isolated from livers. No other agent was isolated using standard aerobic and anaerobic bacteria isolation protocols.

Virology. On Farm A, all six livers from 20-week-old hens submitted immediately after the period of peak of mortality were positive for aHEV helicase gene RNA. In samples collected 3 weeks later only 50% (3/6) of samples from chickens that died and had hepatomegaly were positive for aHEV RNA. On Farm B, all five livers submitted were positive for aHEV helicase gene RNA. Adenovirus DNA was detected in 1 of 2 sample pools from Farm A while all five samples from Farm B were negative.

Sequencing and phylogenetic analysis. For Farm A, two HEV RNA positive samples collected at 20 weeks of age and one sample collected at 23 weeks of age were selected for sequencing. For Farm B, two HEV RNA positive samples collected at 46 weeks of age were sequenced. Sequencing results showed that strains from Farms A and B presented 98.6-100% nucleotide identity for the 361 bp fragment of the helicase gene. Between sequences described here and prototype and avirulent USA strains, there was 89% identity. Between 89.9-90.5% identity was found with genotype 2 strains recovered from healthy chicken flocks in Spain (aHEVSP-46) (Fig. 4). Similarity with genotype 1, 3 and 4 isolates varied between 75.4-83.4%.

Discussion

The present report describes the detection and characterization of aHEV RNA in two layer flocks experiencing clinical disease. Increases in mortality, a drop in egg production in one flock, hepatomegaly, splenomegaly, and hemorrhage into the coelomic cavity were the main features observed, and these findings were consistent with observations previously described for HSS in other field cases (Massi *et al.*, 2005; Agunos *et al.*, 2006; Morrow *et al.*, 2008; Sprygin *et al.*, 2012).

A presumptive diagnosis of HSS can be made on the basis of clinical signs and gross and microscopic lesions (Meng *et al.*, 2013), however, confirmation of aHEV infection requires detection of virus RNA by RT-PCR (Troxler *et al.*, 2011). HSS must be differentiated from hemorrhagic fatty liver syndrome (HFLS) that occurs in caged layers fed high-energy diets and is characterized by accumulation of fat in liver hepatocytes and liver hemorrhages (Agunos *et al.*,

2006). These two syndromes can be differentiated by histological examination, as hepatocytes in HSS livers do not contain excessive lipid and HFLS livers do not have massive necrosis (Agunos *et al.*, 2006). Liver fractures caused by external trauma to the body wall should also be considered as a differential diagnosis. Liver fractures result in massive hemorrhage into the coelomic cavity and coagulated blood can often be observed adhering to the liver surface at the fracture site (Meng *et al.*, 2013). In the present study, no bacterial agents other than *E. coli* were recovered from affected birds and no keel or rib fractures were seen, suggesting that mortality and lesions observed in this flocks were associated with aHEV infection. Although adenovirus DNA was recovered from one sample from Farm A, adenoviruses are commonly found in laying hens and no histopathological lesions suggestive of inclusion body hepatitis were present in any of the studied chickens.

Interestingly, clinical signs of HSS occurred in chickens younger than 20 weeks on Farm A, which is unusual because HSS is typically associated with above-normal mortality in broiler breeder hens and laying hens of 30–72 week of age, with the highest incidence occurring between 40–50 weeks of age (Agunos *et al.*, 2006; Morrow *et al.*, 2008; Sprygin *et al.*, 2012; Meng *et al.*, 2013). Clinical HSS in pullets on Farm A was associated with a two week delay in onset of egg production even though lighting and nutritional programs were the same as those used for other flocks on this farm. Reduced egg production after onset of lay persisted for several weeks before production approached the breed standard. It has been suggested that natural infection of chicken flocks with aHEV occurs around 12 weeks of age (Sun *et al.*, 2004) and subclinical infections appear to be frequent (Sun *et al.*, 2004; Peralta *et al.*, 2009a; Kwon *et al.*, 2012). To the author's knowledge, HSS in young chickens has not been reported to date. HSS observed in pullets in the present case report could have been associated with variation in viral

virulence and tropism, timing and dose of infection, interaction with other management factors, an impaired immune response or additional co-infections. Under field conditions, HSS may also pass unnoticed or be masked by other diseases in pullets.

Differences in timing and dose of infection could partially explain differences in the clinical presentation of HSS in both farms, as no decline in egg production was noticed in Farm B and the rise of mortality in this farm was more subtle compared to what was observed in Farm A. Different clinical presentations of HSS outbreaks have been reported previously, with increases in mortality up to 1.0% a week that may return to previous levels after four weeks or may last until the end of the laying cycle (Agunos *et al.*, 2006; Morrow *et al.*, 2008; Meng *et al.*, 2013). In Farm A, atypical food and water consumption and increased mortality were observed immediately after placement at 16 weeks of age, suggesting that initial aHEV infection may have occurred on the pullet farm prior to moving chickens to the laying house. Infectious avian hepatitis E virus particles have been detected in egg albumen, but vertical transmission has not been confirmed (Guo *et al.*, 2007). Although aHEV transmission within and between flocks appears to occur readily (Meng *et al.*, 2013), clinical signs associated with HSS were limited to one affected house in both farms. Unidentified co-factors in each of these houses may have potentiated and synergized aHEV-induced disease. Lesions and clinical signs associated with HEV have been reported to be dose dependent in non-human primates. Animals infected with a high dose typically progress to significant lesions, whereas animals receiving low doses have subclinical infection without development of hepatic lesions (Tsarev *et al.*, 1994). Dose dependency could partially explain why chickens in only one house in each farm presented clinical signs. Although aHEV could have spread to other flocks in nearby houses on the farm, the number of virus particles circulating in other houses may have been insufficient to induce

significant lesions or clinical signs. However, one limitation of this case study is that chickens from the unaffected flocks on these farms were not investigated for aHEV infection.

Sequences of aHEV obtained in this study shared 99% nucleotide identity in the helicase gene to each other and between 75.4-90.5% identity with previously published sequences. Prior studies reported 76-100% identity among aHEV isolates recovered in different regions of the USA based on partial fragments of the helicase gene (Huang *et al.*, 2002; Sun *et al.*, 2004). Isolates described in this paper clustered with previously reported USA and Spanish genotype 2 isolates.

In this report, characterization of aHEV detected in young and adult layers with HSS on two U.S. egg farms is described. Genetic analysis of the obtained helicase gene sequences showed that the aHEVs in both farms clustered together and were closely related genotype 2.

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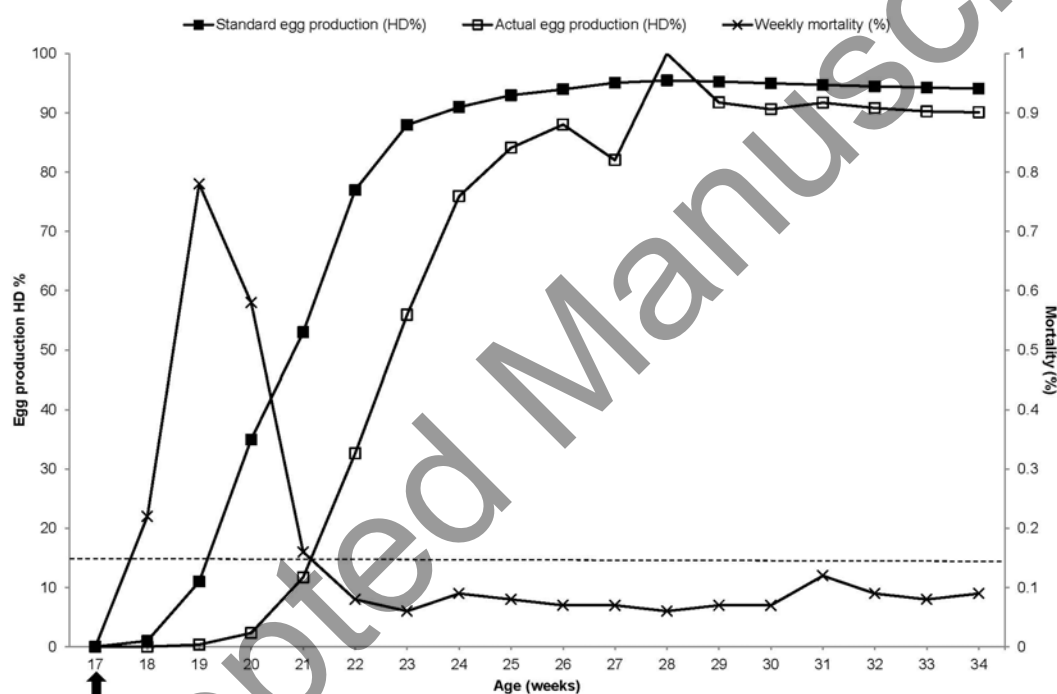
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Figure Legends

Figure 1. Egg production and mortality rates for Farm A (1A) and Farm B (1B). Egg production was compared with the breed standard. The dashed line represents the threshold of a weekly mortality above 0.15%. The black arrow indicates the onset of clinical signs.



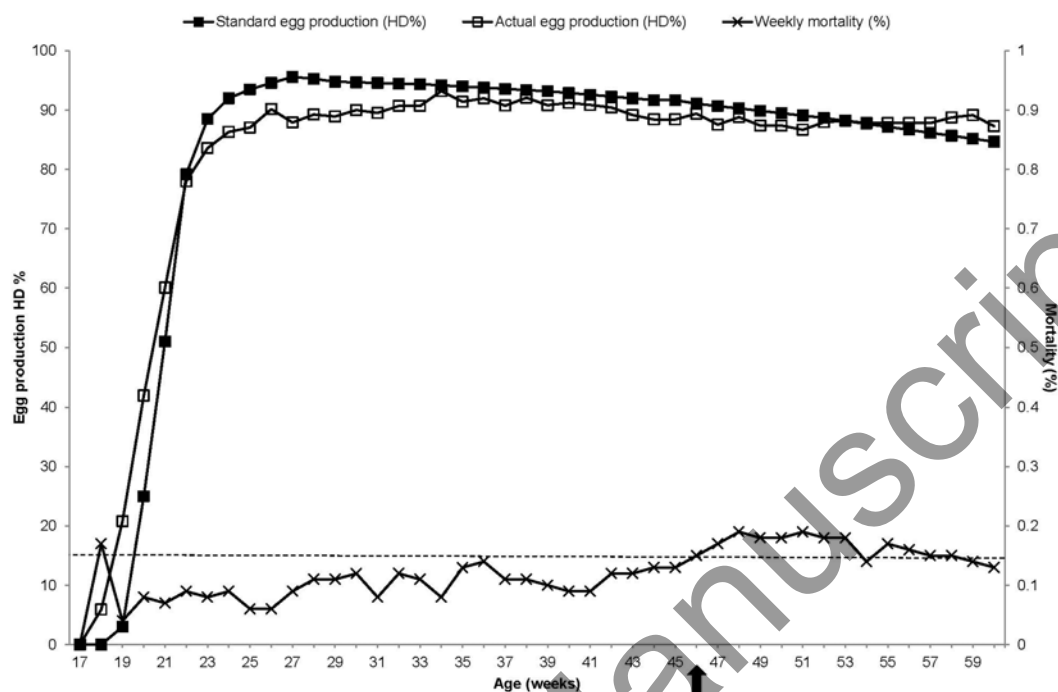


Figure 2. Macroscopic lesions in affected 23-week-old Farm A chickens. 2A: Diffusely enlarged liver. 2B: Congested enlarged liver and yellow foci. 2C: Pectoral muscles; normal (on left) and pale carcass on affected bird (on right).

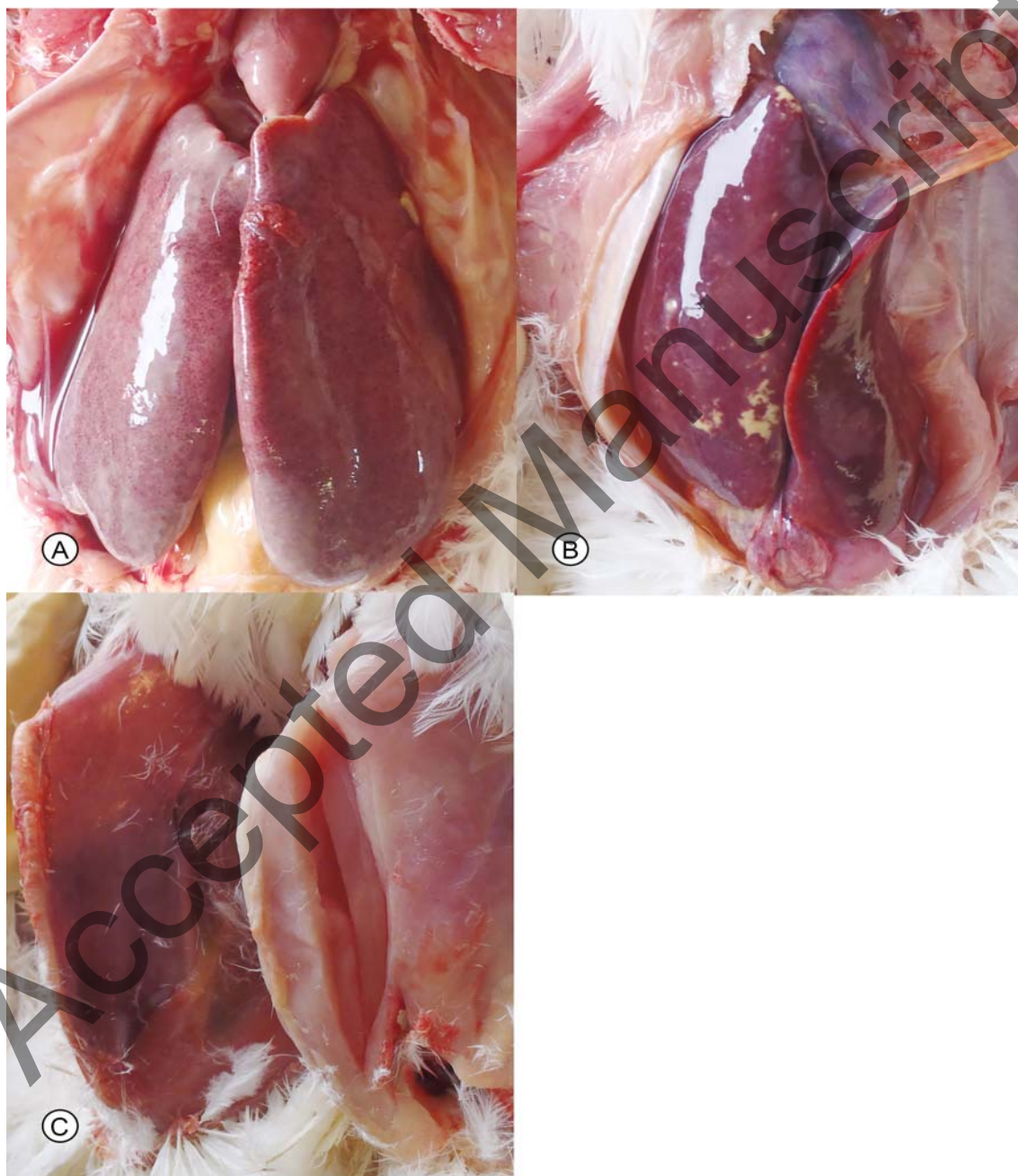


Figure 3. Microscopic liver lesions in affected 23-week-old Farm A chickens. 3A: Intrahepatic hemorrhage (bar= 250 μ m). 3B: Necrosis and fibrin deposition within hepatic parenchyma (bar= 450 μ m). 3C: Heterophils in a portal triad area (bar= 75 μ m). 3D: Vasculitis in a portal triad area (bar= 90 μ m).

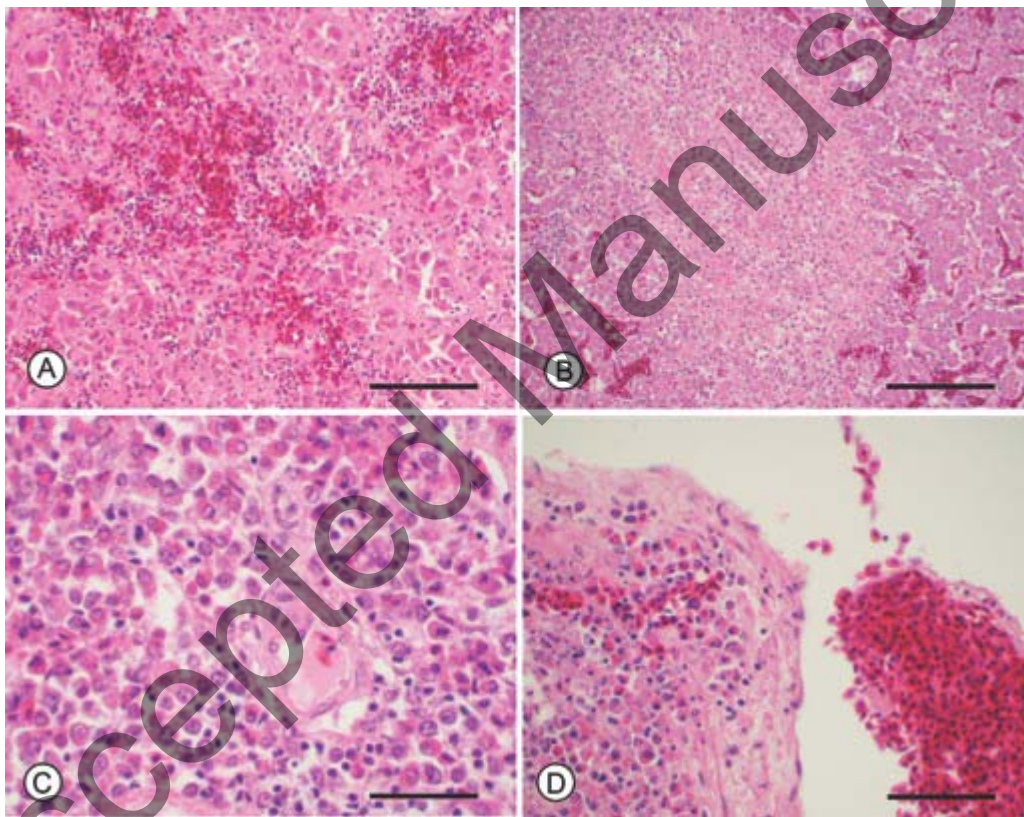


Figure 4. Phylogenetic tree based on 361 bp regions of the helicase gene of aHEV isolates.

Sequences that were obtained in this study are underlined. The phylogenetic tree was constructed by the NJ method. Significant bootstrap values are indicated as a percentage for 1000 replicates. Genotype classification represented as proposed by Marek et al., 2010 and Hsu and Tsai, 2014.

